UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF
PREVENTION, PESTICIDES,
AND TOXIC SUBSTANCES

December 17, 2003

MEMORANDUM

SUBJECT: Transmittal of the Report and Meeting Minutes of the Endocrine Disruptor Methods Validation Subcommittee under the National Advisory Council for Environmental Policy and Technology (NACEPT) held August 18 - 20, 2003.

TO: Dorothy Bowers, Chair
National Advisory Council for Environmental Policy and Technology
Office of Cooperation and Environmental Management
And
Mark Joyce & Sonia Altieri
Designated Federal Officials
National Advisory Council for Environmental Policy and Technology
Office of Cooperation and Environmental Management

FROM: Jane Scott Smith, Designated Federal Official
Endocrine Disruptor Methods Validation Subcommittee
Office of Science Coordination and Policy, OPPTS

THRU: Joseph Merenda, Chair
Endocrine Disruptor Methods Validation Subcommittee
Director, Office of Science Coordination and Policy, EPA

Please find attached the minutes of the NACEPT Endocrine Disruptor Methods Validation Subcommittee Eighth open meeting held in Golden, CO. from August 18 - 20, 2003. This meeting summary covers the status/results of the prevalidation work on the fish screening assay, specifically: the survey of vitellogenin methods in Fathead Minnow, Zebrafish and Medaka; the comparative evaluation of the Fathead Minnow assays; and the Fish Screen (non-spawning) assay; and the steroidogenesis assay optimized protocol. Also to provide input and advice on the EDSP's validation plans for the fish screening assay, steroidogenesis assay, strain/species white paper, chemicals used in EDSP's prevalidation and validation; avian detailed

review paper; issues related to the pubertal assays; and an update on the amphibian workshop conducted recently.

Information about this NACEPT EDMVS meetings and activities can be obtained from the website at http://www.epa.gov/scipoly/oscpendo or the OPPT Docket, Docket Number OPPT-2003-0027 online (www.epa.gov/edocket) or at (202) 566-0280. Interested persons are invited to contact Jane Smith, EDMVS Designated Federal Official (DFO), via e-mail at smith.jane-scott@epa.gov.

cc: Charles Auer, OPPT
Daiva Balkus, OCEM
Sandra Evalenko, OPPTS
Elaine Frances, ORD
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Joseph Merenda, OSCP
Margaret Schneider, OPPT
Adam Sharp, OPPTS
OPPT Docket # OPPT-2003-0027

REPORT
OF
ENDOCRINE DISRUPTOR METHODS VALIDATION SUBCOMMITTEE MEETING
A Subcommittee of the National Advisory Council for
Environmental Policy and Technology
August 18 – 20, 2003
AT
Table Mountain Inn; 1310 Washington Avenue
Golden, CO 80401

This meeting was a review and discussion of the results and status of the prevalidation work on the fish screening assay, specifically: the survey of vitellogenin methods in Fathead Minnow, Zebrafish and Medaka; the comparative evaluation of the Fathead Minnow assays; and the Fish Screen (non-spawning) assay; and the steroidogenesis assay optimized protocol. Also to provide input and advice on the EDSP's validation plans for the fish screening assay, steroidogenesis assay, strain/species white paper, chemicals used in EDSP's prevalidation and validation; avian detailed review paper; issues related to the pubertal assays; and an update on the amphibian workshop conducted recently.

Jane Scott Smith, DFO	**Joseph Merenda, Chair**
Endocrine Disruptor Methods	**Endocrine Disruptor Methods**
Validation Subcommittee under	**Validation Subcommittee under**
The National Advisory Council for	**The National Advisory Council for**
Environmental Policy and Technology	**Environmental and Technology**
Date: _____	**Date: _____**

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EDMVS Members in Attendance at the August 2003 Meeting

Joseph Merenda, Chair
U.S. EPA

William Benson, Ph.D., Vice Chair
U.S. EPA

Theodora Colborn, Ph.D
World Wildlife Fund

Robert D. Combes, Ph.D.
Scientific Director, FAME

Peter L. deFur, Ph.D
Commonwealth University

James T. Stevens, Ph.D.
Wake Forest U. School of Medicine

David Hattan, Ph.D.
Food and Drug Administration

Robert J. Kavlock, Ph.D.
U.S. EPA

Timothy Kubiak, M.P.A.
U..S. Fish and Wildlife Service

Shane Snyder, Ph. D.
Southern Nevada Water Authority

Rodger Curren, Ph.D.
Institute for In Vitro Sciences, Inc.

Gerald A. LeBlanc, Ph.D
North Carolina State University

Ron Miller, Ph.D
The Dow Chemical Company

Susan C. Nagel, Ph. D.
U. Missouri - Columbia

James W. "Willie" Owens, Ph.D.
The Procter & Gamble Company

Thomas L. Potter, Ph.D.
USDA- Agriculture Research Service

William Stokes, D.V.M.
NIEHS

Glen Van Der Kraak, Ph.D.
University of Guelph

Mildred Christian, Ph. D.
Argus International

Facilitator
Paul De Morgan
RESOLVE

Designated Federal Official
Jane Scott Smith
Office of Science Policy and Coordination

Presenters

In Order of Presentation

August 18, 2003

Jane Smith, DFO
EPA, OSCP

Mr. Michael Blanton
Battelle

Dr. Irvin Schultz
Battelle

Dr. Hirofumi Yokota
Chemicals Evaluation and Research Institute (CERI), Japan

August 19, 2003

Dr. Les Touart
EPA, OSCP

Dr. Sherry Parker
Research Triangle Institute (RTI)

Dr. Jimmy Spearow
University of California, Davis

Gary Timm
EPA, OSCP

August 20, 2003

Dr. Les Touart
EPA, OSCP

Dr. L. Earl Gray, Jr.
EPA, Office of Research & Development (ORD)

Carol Sloan
Research Triangle Institute (RTI)

Gary Timm
EPA, OSCP

Oral Public Comment

In Order of Presentation:

August 18, 2003

Rick Becker, Ph.D.
American Chemistry Council (ACC)

Tilghman Hall
Bayer CropScience for Crop Life America

Katie Holmes
BASF Agro Research for Crop Life America

Reinhard Fischer
Bayer CropScience for Crop Life America

August 19, 2003

Rick Becker, Ph.D.
American Chemistry Council (ACC)

David Paulsen
Clark County Water Reclamation District, Las Vegas, NV

Jim Pletel
Hampton Road Sanitation District

NOTICE

This meeting summary has been written as part of the activities of the National Advisory Council on Environmental Policy and Technology (NACEPT), Endocrine Disruptor Methods Validation Subcommittee (EDMVS). This meeting summary has not been reviewed for approval by the United States Environmental Protection Agency (Agency) and, hence, the contents of the meeting summary do not necessarily represent the views and policies of the Agency, nor of other agencies in the Executive Branch of the Federal government, nor does mention of trade names or commercial products constitute a recommendation for use.

The NACEPT EDMVS was established in partial fulfillment of a Congressional statute. When Congress amended the Federal Food Drug and Cosmetics Act (FFDCA) in the Food Quality Protection Act (FQPA) of 1996, it directed the U.S. Environmental Protection Agency (EPA) to develop a screening program to determine whether certain substances may have hormonal effects in humans. To ensure that EPA has the best and most up-to-date advice available regarding the validation of the screens and tests in the EDSP, EPA established the Endocrine Disruptor Methods Validation Subcommittee (EDMVS) under the NACEPT. The EDMVS provides independent advice and counsel to the Agency through NACEPT on scientific and technical issues related to validation of the EDSP Tier I and Tier II assays, including advice on methods for reducing animal use, refining procedures involving animals to make them less stressful, and replacing animals where scientifically appropriate. The EDMVS held their first meeting in October of 2001. This was the eighth meeting of the EDMVS.

The August 18 - 20, 2003 open meeting of the EDMVS was announced in the Federal Register on July 30, 2003 (Volume 68, Number 146). Further information about NACEPT EDMVS meetings and activities can be obtained from its website at http://www.epa.gov/scipoly/oscpendo or the OPPT Docket number OPPT-2003-0027 online at www.epa.gov/edocket or at (202) 566-0280. Interested persons are invited to contact Jane Smith, EDMVS Designated Federal Official (DFO), via e-mail at smith jane-scott@epa.gov.

National Advisory Council for Environmental Policy and Technology (NACEPT)
Endocrine Disruptor Methods Validation Subcommittee (EDMVS)
Plenary Meeting
August 18 – 20, 2003

Table Mountain Inn
1310 Washington Avenue
Golden, CO 80401
(303) 216-8040

Proposed Agenda

Meeting Objectives:

1. Review and discuss the status/results of the prevalidation work on:
   - the fish screening assay, specifically: the survey of vitellogenin methods in Fathead Minnow, Zebrafish and Medaka; the comparative evaluation of the Fathead Minnow assays; and the Fish Screen (non-spawning) assay; and
   - the steroidogenesis assay optimized protocol.
2. Provide input and advice on the:
   - EDSP's validation plans for the fish screening assay and steroidogenesis assay;
   - strain/species white paper;
   - chemicals used in EDSP's prevalidation and validation.
   - avian detailed review paper; and
   - issues related to the pubertal assays.
3. Receive an update on the amphibian workshop conducted recently.

Monday, August 18, 2003

1:30 – 1:40 **Welcome and Opening Comments**
Joe Merenda, EDMVS Chair and Director, Office of Science Coordination and Policy (OSCP), EPA

1:40 – 2:00 **Introduction, Agenda Review, and Review of Previous Meeting Summary**
Paul De Morgan, Facilitator, RESOLVE

2:00 – 2:30 **Review of EDMVS Work Plan**
Jane Smith, EDMVS Designated Federal Official, OSCP, EPA

2:30 – 3:45 **Presentation on Fish Screening Assays:**
- **Results of the Survey of Vitellogenin Methods in Fathead Minnow**
- **Results of the Survey of Vitellogenin Methods in Zebrafish and Medaka**

Michael Blanton, Battelle

3:45 – 4:00 **Break**

4:00 – 4:45 **Presentation on Fish Screening Assay: Results of the Comparative Evaluation of the Fathead Minnow**
Dr. Irvin Schultz, Battelle

4:45 – 5:15 **Presentation on OECD Phase 1A: Feasibility to Demonstrate the Fish Screen (Nonspawning) Method**
Dr. Hirofumi Yokota, Chemicals Evaluation and Research Institute (CERI), Japan

5:15 – 5:45 **Public Comment**
Members of the public will be given an opportunity to comment on any aspect of the EDMVS work. The amount of time given to each individual will depend on the number of people wishing to provide comment.

5:45 **Adjourn**

Tuesday, August 19, 2003

7:45 – 8:00 **Settling In**

8:00 – 9:30 **Discussion of the Fish Screening Assay**

9:30 – 10:00 **Overview of Amphibian Workshop**
Dr. Les Touart, OSCP, EPA

10:00 – 10:15 **Break**

10:15 – 12:30 **Presentation and Discussion on Strain/Species**
Dr. Sherry Parker, Research Triangle Institute (RTI)
Dr. Jimmy Spearow, AFFILIATION?

12:30 – 1:30 **Lunch**

1:30 – 2:30 **Update on Chemicals Used for EDSP's Prevalidation and Validation**
Gary Timm, OSCP, EPA

2:30 **Adjourn**

Wednesday, August 20, 2003

8:00 – 8:15 **Settling In**

8:15 – 10:15 **Presentation and Discussion of Avian Detailed Review Paper**
Dr. Les Touart, OSCP, EPA

10:15 – 10:30 **Break**

10:30 – 12:00 **Issues Related to the Pubertal Assay**

Dr. L. Earl Gray, Office of Research and Development, EPA

12:00 – 1:00 Lunch

1:00 – 1:30 Presentation on Steroidogenesis Assay Optimized Protocol
Carol Sloan, RTI

1:30 – 2:30 Presentation and Discussion on Steroidogenesis Prevalidation Study Plan
Gary Timm, OSCP, EPA

2:30 – 2:45 Break

2:45 – 3:15 Public Comment
Members of the public will be given an opportunity to comment on any aspect of the EDMVS work. The amount of time given to each individual will depend on the number of people wishing to provide comment

3:15 – 3:30 Next Steps and Agenda for Next Meeting

3:30 Adjourn

Introduction

The Office of Science Policy and Coordination's Endocrine Disruptor Screening Program established the Endocrine Disruptor Methods Validation Subcommittee (EDMVS) under The National Advisory Council for Environmental Policy and Technology (NACEPT). The first EDMVS meeting was held in October 2001. That initial meeting brought the members together to review the mission statement and discuss subcommittee roles and responsibilities. The second meeting, held in December 2001, was the first time the subcommittee members were presented with specific questions regarding assay protocols. This third meeting, held March 2002, continued discussions on protocols as well as some discussions on the validation process, Core Chemicals, 'low dose' and means of assessing human health effects. The fourth meeting, held as a teleconference, was wholly concerned with the Steroidogenesis assay. The fifth meeting held July 23-24, 2002, was concerned with screening criteria, core chemicals, In Vitro ER/AR assays, and dose setting as well as test results of two special studies, a pubertal study involving restricted feeding, and a mammalian 2-generation study involving PTU. Detailed review papers were presented on amphibian metamorphosis and invertebrate assays. The sixth meeting, held as a teleconference, was to receive comments and advice on the Fish Lifecycle DRP (Tier II). The seventh meeting was held June 5 – 6, 2003 to review and discuss prevalidation results for the steroidogenesis assay, aromatase assay and the mammalian two generation assay as well as the validation plans for each.

This eighth meeting held August 18 – 20, 2003 reviewed and discussed the status/results of the prevalidation work on:

- the fish screening assay, specifically: the survey of vitellogenin methods in Fathead Minnow, Zebrafish and Medaka; the comparative evaluation of the Fathead Minnow assays; and the Fish Screen (non-spawning) assay; and
- the steroidogenesis assay optimized protocol.

Provided input and advice on the:

- EDSP's validation plans for the fish screening assay and steroidogenesis assay;
- strain/species white paper;
- chemicals used in EDSP's prevalidation and validation.
- avian detailed review paper; and
- issues related to the pubertal assays.

Receive an update on the amphibian workshop conducted recently.

Endocrine Disruptor Methods Validation Subcommittee (EDMVS)
Eighth Plenary Meeting
August 18-20, 2003

Meeting Summary

On August 18-20, 2003, the U.S. Environmental Protection Agency (EPA) convened the eighth meeting of the EDMVS. The meeting objectives included

4. Review and discuss the status/results of the prevalidation work on
   - the fish screening assay, specifically: the survey of vitellogenin methods in fathead minnow, zebrafish and medaka; the comparative evaluation of the fathead minnow assays; and the fish screen (non-spawning) assay; and
   - the steroidogenesis assay optimized protocol.
5. Provide input and advice on
   - EDSP's validation plans for the fish screening assay and steroidogenesis assay;
   - strain/species white paper;
   - chemicals used in EDSP's prevalidation and validation.
   - avian detailed review paper; and
   - issues related to the pubertal assays.
6. Receive an update on the amphibian workshop conducted recently.

Copies of presentation slides and other materials distributed at the meeting may be obtained by contacting Jane Smith at smith.jane-scott@epa.gov or 202/564-8476. Many of the materials also are available on the EPA website at http://www.epa.gov/scipoly/oscpendo/ . EPA has established an administrative record for this meeting under docket control number OPPT- 2003 – 0027. The docket is available for inspection in the TSCA Nonconfidential Information Center, 1201 Constitution Ave. N.W., Washington, DC. The center is open from noon to 4 p.m., Monday through Friday, excluding legal holidays. The telephone number of the center is (202) 566-0280.

I. Welcome and Opening Comments

Joe Merenda, director of the EPA Office of Science Coordination and Policy (OSCP) and chair of the EDMVS, welcomed the EDMVS and members of the public. He acknowledged the large amount of work and materials put before the EDMVS.

Jane Smith, designated federal official for the EDMVS, reminded the EDMVS and members of the public that the committee must follow the Federal Advisory Committee Act (FACA) rules for meeting procedures, including public comment.

II. Introductions, Agenda Review, and Review of Previous Meeting Summary

Mr. Merenda asked the EDMVS members to identify themselves and their organizations (see attachment A).

Paul De Morgan, senior mediator with RESOLVE, reviewed the meeting agenda and groundrules. He informed the EDMVS that the June meeting summary is not yet ready for distribution, but will be sent to members soon for their review.

III. Review of EDMVS Work Plan

Ms. Smith described the progress of the Endocrine Disruptor Screening Program (EDSP) and provided the status of each assay in tier 1 and tier 2. (As indicated above, copies of slides from Ms. Smith's presentation may be obtained from the docket or EPA website.) She outlined likely topics for the December 2003 EDMVS meeting, including male and female pubertal assays, in utero through lactation assay, update on androgen receptor binding, aromatase, and adult male assay. For the spring 2004 timeframe, EPA expect to be prepared to discuss the amphibian detailed review paper (DRP), amphibian screen transferable protocol, fish screen multi-chemical results, Organization for Economic Cooperation and Development (OECD) phase 1B, and the mysid transferable protocol.

Ms. Smith explained that EPA is in the process of selecting members for the subcommittee when it is rechartered in the fall. An orientation for new members is tentatively planned in conjunction with the next meeting (should reselection be completed.).[1]

IV. Fish Screening Assays: Comparative Evaluation of Vitellogenin Methods

Michael Blanton, Battelle, presented a summary of the comparative evaluation of vitellogenin (VTG) methods for fathead minnow, medaka, and zebrafish. (As indicated above, copies of slides from Mr. Blanton's presentation may be obtained from the docket or EPA website.) He explained that the purpose of the studies was to conduct a survey of existing VTG analytical methods for suitability in a routine screening program; the studies were not intended to be a method validation.

Mr. Blanton described three methods for measuring VTG induction in fishes: enzyme-linked immunosorbant assays (ELISA), messenger ribonucleic acid (mRNA) detection, and mass spectometry. He outlined the objectives and methods for the fathead minnow study and the zebrafish and medaka study and summarized the results of each study. In closing, he shared the following discussion points and recommendations:

- Various studies have demonstrated the utility of VTG as a biomarker indicating endocrine disruption in some types in fish.
- Based upon the present results, most laboratories and methods considered are capable of distinguishing changes in VTG levels in fathead minnow, zebrafish, and/or medaka.

[1] NOTE: EPA was in the process of reselecting the EDMVS membership when NACEPT recommended that the EDMVS become an independent Committee, because of the highly specialized nature of its mission. In light of this new direction, EPA is in the process of determining the best course of action. Members will be kept posted on the progress.

- Issues need to be resolved before VTG measurement could be used as a reliable screening and testing tool. In particular, intra-assay variability was high.

- Recommendations:
  - Develop specific performance criteria for VTG analytical methods.
  - Use a single, standardized protocol for each fish species to quantify VTG in interlaboratory validation trials.

Discussion

One member inquired whether all the methods showed similar trends in induced versus uninduced males. Mr. Blanton responded that a relative trend could be seen in the evaluation, but not a statistical trend. In response to a question about which method appeared to be the most promising and consistent, Mr. Blanton added that the method results were variable and depended on the ability to get male fish to produce VTG. The findings do show a relative increase in the concentration of protein in fish.

In response to other questions about inter-lab variability, intra-lab variability, and methodology, Irvin Shultz, Battelle, clarified that each lab was provided three replicates of each sample. He indicated that samples for the four conditions (uninduced male, induced male, uninduced female, and induced female) were derived from a single homogeneous pool for each respective condition.

Members commented on the relative difference in magnitude of the absolute values in VTG concentrations between labs. Mr. Blanton explained that the evaluators did not attempt to investigate these differences, but possible explanations include sample degradation through preparation, shipping, or handling. One member asked whether sufficient spread between the induced and uninduced samples existed to determine decreases statistically. Mr. Blanton replied that in the case of females, this is possible, depending on their reproductive state.

A member inquired whether the labs are proficient in conducting the assay. Mr. Blanton answered that the labs have indicated that they are capable of conducting the assays.

Members asked that the presenters provide a summary table of the descriptions of the various methods in relation to spikes in the data. The presenters offered to locate tables in the report that could provide this information. They also clarified that the evaluation used uninduced males as positive controls. A member followed up by asking if there was an attempt to look at the absolute concentration of the positive control or whether data had been adjusted to that positive control.

A member asked if Battelle has developed any recommendations as a result of the assay comparison. The presenters commented that they focused on a survey of different measures rather than development of specific recommendations, but did note that in comparing liver, whole body, and plasma data, plasma analysis may be preferable if researchers could obtain plasma from all three species.

V. **Fish Screening Assays: Comparative Evaluation of the Fathead Minnow Assays**

Dr. Schultz presented a summary of the comparative evaluation of fathead minnow assays for detecting endocrine-disrupting chemicals. (As indicated above, copies of slides from Dr. Schultz's presentation may be obtained from the docket or EPA website.) He explained that the purpose of this study was to a) evaluate the transferability and sensitivity of the short-term reproduction assays with the fathead minnow to identify specific modes of action and b) conduct a side-by-side comparison of the 21-day assay with two separate 14-day assays.

Dr. Schultz outlined the study methods and summarized the EPA 21-day assay, the 14-day nonspawning assay, and the EPA 14-day assay. He reviewed the study results for the four test chemicals: methoxychlor, trenbolone, flutamide, and fadrozole. He noted the major conclusions of the study for each of the chemicals and reported that the EPA14-day spawning protocol is the suggested protocol. He also shared the study recommendations for further research in two areas:

- additional development of the abbreviated EPA14-day spawning protocol to evaluate how well the assay performs in comparison to the full 21-day protocol with weaker acting and high log-P compounds; and
- work on endpoint measurement techniques to standardize and reduce variability.

Discussion

A member inquired how the labs used a proportional diluter and whether chemical buildup in the system was found. Dr. Shultz answered that the diluter is used to automatically prepare the mixture of toxicant waters. Stability tests are conducted prior to the assays and the concentration in the tanks is measured to check delivery of the desired concentration of chemical.

Members requested more information on how the infections in the methoxychlor fish experiment seen in the evaluation influenced the results. The presenters indicated that the infections could have resulted from females' exposure to mycobacteria or being carriers of bacteria when purchased, but the precise cause is unclear. A member pointed out that male mortality in the methoxychlor experiment could be responsible for reduced fecundity. Dr. Shultz added that the fecundity analysis accounts for a loss in replicates. Another member commented that if high doses of a chemical led to early mortality, this could impact female egg production and, in combination with the infections in females, raise problems with the results of the experiment. The presenters responded that working with younger fish not yet at reproductive age when shipped could reduce some of these issues.

A member asked for clarification of the summary statistical results. The presenters explained that the values of VTG were not significantly different from the controls, that the sample size was small, and the results showed absolute values of VTG in the high range. They added that fecundity appeared to be a sensitive endpoint. In response to a request for comparisons among different assays and chemicals, the presenters directed members to the report summary, which highlights the statistically significant responses. Ms. Smith reminded members that the appendix that contains additional statistics is available from her upon request.

One member questioned the use of the term "corpora lutea" in reference to fish histology, as it applies only to mammals. The structure studied was, perhaps, the "post-ovulatory follicle."

VI. OECD Phase 1A: Feasibility to Demonstrate the Fish Screen (Nonspawning) Method

Hirofumi Yokota, Chemicals Evaluation and Research Institute (CERI), Japan, presented the draft results of phase 1A work of the Organization for Economic Cooperation and Development (OECD) on a non-spawning fish assay. (As indicated above, copies of slides from Dr. Yokota's presentation may be obtained from the docket or EPA website.) He outlined the assay protocol and explained that the aim of the phase 1A validation study was to obtain information on a) the relevance of the endpoints used in terms of their sensitivity and (species) specificity and b) the reproducibility of test results. He reviewed the draft results of the study for gross morphology (secondary sexual characteristics and gonado-somatic index (GSI)) and VTG levels, noting that the results for gonad histology are not yet available. He summarized the draft results as follows:

- GSI
  - Significant differences were not always observed in all studies.
  - The values in both intra- and inter-laboratory varied considerably possibly due to differences in sexual maturity.
  - Some female medaka and zebrafish spawned during exposure, indicating that the method of isolating the sexes was not adequate.
- Secondary Sexual Characteristics (fathead minnow and medaka)
  - Significant differences were not always observed in all studies.
  - The quantification method was not standardized between laboratories.
- VTG
  - Significant differences were observed in all studies.
  - The values in both the control and treatment groups differed with the ELISA kits.
  - The preparation methods of the samples were not standardized.

Dr. Yokota noted that the draft report will be submitted to OECD by September 1st and then circulated to the members of the OECD Fish Drafting Group. The final results will be discussed at the OECD Fish Drafting Group meeting on October 21st and 22nd.

Discussion

A member inquired about the delivery of materials to the labs and how the dose was administered. Dr. Yokota responded that the exposure system varied among the labs, but generally a stock solution was used and then diluted. The labs used a flow-through system in which there is a continuous flow of solution at a constant concentration. The dose and the method of administration was the same for all labs.

Another member asked if the experimenters noted any abnormal responses and if these were correlated to particular endpoints. Dr. Yokota answered that mortality was observed in the highest concentrations of chemicals, but abnormal responses observed were not dose related. He also clarified that only healthy fish were used in the experiment, based on observations of health during acclimation.

A member requested clarification on the purpose of the study: are researchers looking for the most sensitive response or the most reproducible response? Dr. Touart responded that the

purpose needs clarification through further discussion and reminded members that the phase 1A study is designed as a feasibility study to identify core endpoints for reproducibility and sensitivity of detecting endpoints.

In response to a question regarding why the findings show such variability in VTG induction between different species, Dr. Yokota answered that the differences among strains of zebrafish need to be examined. Potential problems with sample collection also could have influenced variability.

A member commented that further discussions of general standards and procedures for experiments with non air-breathing animals are needed, as well as further examination of immune suppression and disease susceptibility as an endpoint versus a secondary consequence of exposure to the test chemical.

Another member asked what histological endpoints were examined in each species. Dr. Yokota answered that a round-robin evaluation of histology is being conducted with readers blinded to treatment up to a point to determine abnormalities. A discussion of which endpoints to standardize is currently under discussion.

VII. **Discussion of the Fish Screening Assays**

Dr. Touart provided context and background for the fish screening assays presented at the meeting. In March 2002, the OECD Fish Drafting Group issued recommendations for study plans for VTG and fish comparison. The Fish Drafting Group will meet again in October 2003 to consider its next phase of validation work for fish assays. Questions the group will consider include whether a standardization of VTG measurement methods is needed, quantitative comparisons, and the reproducibility of methods. The U.S. agreed to initiate a survey of methods, the results of which indicate that standardization may be merited. The EDMVS will discuss the assays and provide advice to EPA to help the agency prepare its comments for the OECD Fish Screening Group. Dr. Touart noted that for example, the EDMVS may want to comment to EPA on whether the Fish Drafting Group should focus on a particular assay, which path to take, and which species to use. Other considerations are that the assays should be fully reproducible and relevant to the screening.

Dr. Touart also commented that members should think about what information is needed to guide discussions, including what existing data should be reworked and what new data could help inform comparisons.

Discussion

A small group of EDMVS members created three broad questions to help frame the discussion of the fish screening assay:

1) Should EPA continue to move forward with the fish screening assay?

Those in favor of proceeding offered the following comments:

- The assay is useful to fill potential gaps.
- Fish are uniquely sensitive.
- The assay is reliable.
- Powerful data have been presented; the assay should move forward.
- The in vivo test recognizes the unique physiology of the fish and based on that physiology the fish may be uniquely sensitive to chemicals.

Suggestions from members of improvements and considerations to address before moving forward included the following:

- Reassess biological significance.
- Document rationale.
- Develop and document prediction model.
- Focus on fewer species – use fathead minnow.
- Narrow the focus.
- Look at more than VTG.
- Use of fecundity as an endpoint.
- Use ELISA methods.
- Focus on addressing statistical problems in the presentation of results, not testing methodology.
- Hold off on considering methoxychlor until the tests have been rerun under conditions identical to those for the other three experimental chemicals.
- Does the fish assay add value in tier 1? Is there a need for more information on fish endocrine disruptors?

Those not in support of moving forward noted the following:

- The methods are not ready for prevalidation.
- Explore other ways to measure VTG that could be easier to use.
- The assay is not worthwhile; it requires too much work.
- This assay may not achieve the goal to trigger tier 2 for fish.
- There is no need for the 14-day assay because of cost and animal care issues.
- False negatives are a problem.
- Work is needed to resolve the technical problems of measuring the parameters, particularly the variation.

2) *If so, can current data be used to narrow the focus of the assay?*

A member suggested comparing laboratory data on exposed and unexposed males and females.

3) *What additional data are needed to recommend which endpoints, methodologies, etc., to include in the assay, and how should EPA move forward to collect the data?*

Additional data suggested included

- Comparisons of reductions in fecundity across chemicals.
- VTG comparisons and stability of proteins.
- In vitro screening assays to compare performance.
- Data on sources of variation, including the accuracy of the assay.

- Histology.
- Biostatistical review/distribution.
- Power and baseline variability across assays.

Members offered the following specific comments on endpoints:

- Use one mechanistic endpoint.
- Look at the way fish are exposed at their gills, detoxification properties, and egg laying properties to ensure differences in vulnerability are considered in decision to move forward.
- Start with more endpoints, then reduce after deciding which are the critical endpoints.
- Reduce endpoints only after validation studies.

General Comments

Members offered a number of other comments on test methodology:

- Define how EPA will use results when the animals have infections.
- Define dose selection in future studies, including criteria and parameters.
- Solubility is a critical parameter.
- Use enough negative materials to define specificity.
- Use non-specific toxics to get at specificity.
- Standardize pathology nomenclature.
- Performance measures are needed to develop a standard protocol.
- Move forward with one species for now and use that as a basis for establishing performance standards that can be applied to other species. Make sure to follow the same protocol with other species so comparisons can be made.
- Get the tier 1 screening battery in place to collect data, then refine the assays.
- Inconsistency among labs is a practical difficulty that demonstrates the need for consideration of animal husbandry and procurement procedures.
- Do not exclude in vitro assays; consider the European Centre for the Validation of Alternative Methods (ECVAM) workshop report before making decisions.
- Be careful when using terms such as sensitivity and specificity, as these terms have specific meanings in the context of validation studies.
- A table that breaks out each laboratory and technique would be helpful to show the capability of each technique.

Battelle representatives suggested other options for looking at variation in results and statistical significance, such as looking at data by replicate, matching endpoints with fish, and conducting additional statistical analysis. Battelle will look at additional statistical analysis to get further clarity on variation in results versus statistical variations.

A member inquired about how well the current results compare with the Duluth study in reproducibility and endpoints studied. Dr. Shultz explained that researchers used information from the Duluth study to choose the dose for the assays.

VIII. **Overview of Amphibian Workshop**

Leslie Touart, EPA, presented an overview of the International Workshop on the Use of Anuran models for Endocrine Disruption and Reproductive Toxicology. (As indicated above, copies of slides from Dr. Touart's presentation may be obtained from the docket or EPA website.) He reported that experts from eleven countries participated in one day of presentations on general endocrinology, reproductive biology, and test methods for screening and testing, and in one day of breakout group discussions on screening assays and testing methods for thyroid axis disruption, diagnostic indicators of amphibian endocrine disruption, and reproductive endocrinology and testing. He then summarized the key topics of discussion.

Dr. Touart reported that the OECD Amphibian Expert Group met following the workshop. The main objectives of the meeting were to a) discuss and agree on the preferred approach for a frog metamorphosis assay for the detection of thyroid disruptors, b) discuss and agree on the main parameters of a protocol, and c) agree on the time frame and action plan for further work in the coming months. Dr. Touart shared information from the group's work on identifying thyroid-related modes of action, possible endpoints, and relevant chemicals for evaluating assays. He identified which countries have conducted the amphibian metamorphosis assay with which chemicals and presented a table summarizing measurement techniques, priority level, and research needs for various endpoints.

Dr. Touart reported that a protocol optimization is underway, comparing a protocol beginning with the stage 51 animals and continuing for 21 days with a protocol using animals at stage 54 and continuing for just 14 days. The optimization is expected to be complete in December 2003. It is expected that from the results of this work, a draft protocol would be prepared for validation in 2004.

Discussion

Dr. Touart clarified that the amphibian thyroid screen would be used as a tier 1 assay, with key endpoints of thyroid histology and developmental markers. EPA plans to bring a proposal for a protocol to the EDMVS in the spring of 2004, including plans for a multichemical evaluation.

A member recommended that EPA keep the thyroid screen short and simple. The member also suggested beginning with one or two chemicals during inter-laboratory testing before involving many substances. Once it is determined that the test works with a few chemicals, then validation should proceed with 4 - 5 positives and 1- 2 negatives. It was suggested that two chemicals for validation is far too limiting. Dr. Touart noted that EPA will make decisions on the number of compounds following decisions on the protocol. Dr. Touart also clarified that the inter-laboratory exercise scheduled for summer 2004 includes both prevalidation and validation efforts.

Another member encouraged EPA to adopt OECD terms when discussing the various steps of the work.

IX. **Strain/Species**

Sherry Parker, RTI International, presented a summary of the *White Paper on Species/Strain/ Stock in Endocrine Disruptor Assays*. (As indicated above, copies of slides from Dr. Parker's presentation may be obtained from the docket or EPA website.) The purpose of the paper was to summarize the interspecies and intraspecies similarities and differences in response to endocrine endpoints that are being considered in proposed EDSP assays, in order to determine whether specific species/strains should be preferred or avoided when screening for endocrine activity. Dr. Parker outlined the literature search strategy and scope of the paper. She summarized the agent- and endpoint-specific intraspecies differences and the rat interstrain comparisons. She explained that determining interspecies similarities and differences is difficult due to the paucity of studies comparing the effects of endocrine disrupting chemicals in more than one species and due to the high variability across strains within a species.

In closing, Dr. Parker shared the following conclusions:

- Comparisons revealed variability in effects produced by endocrine-disrupting chemicals on endocrine endpoints from strain to strain. Endocrine effects were chemical specific, strain specific, endpoint specific, and, in some cases, laboratory specific. There were more sensitive and less sensitive strains to endocrine-active compounds (EACs) among both outbred and inbred strains, depending on the chemical used and the endpoints evaluated.
- Inbred strains are homogeneous at all loci, and have a limited range of responses (less variability, but an effect may be missed), so using several genetically defined inbred strains in endocrine screens may be the only way to provide a broad spectrum of responsivity. If selecting a single strain for endocrine screens, outbred strains have more genetic variability, exhibit a broader range of responsivity (with a greater likelihood of detecting an effect), and may be more appropriate. Outbred strains, which are heterogeneous like humans and other species of interest, may provide a more appropriate animal model for determining the effects of EACs.
- Since the actions of EACs were generally observed for more than one endpoint, there is a greater likelihood of detecting an endocrine disruptor in a study with many endpoints.
- In current OECD and EPA validation efforts for the Uterotrophic and Hershberger Assays (looking at many of the same endpoints), there was no effect on responsivity of different strains (housing, feed, bedding, etc.) with potent androgens and estrogens.

Jimmy Spearow, University of California at Davis, who reviewed the white paper, shared a presentation outlining points on which he did not concur with the white paper. (As indicated above, copies of slides from Dr. Spearow's presentation may be obtained from the docket or EPA website.) He reviewed the specific information that led him to conclusions different from those in the white paper and then summarized his conclusions, including the following:

- The use of isogenic strains rather than outbred strains in the EDSP will provide more precise and reproducible bioassays for detecting endocrine disrupting activities.
- Given the finding of many strain-specific differences by endocrine-disrupting-agent interactions, and no single optimal strain for detecting all endocrine agents, conducting EDSP assays with multiple strains on different isogenic genetic backgrounds would better insure that all the animals tested are not resistant to the endocrine disrupting chemical (EDC) being analyzed. Otherwise, the use of a single strain, especially one which is genetically resistant to certain EDCs in the EDSP, risks underestimating effects of EDCs on sensitive genotypes.

Discussion

In response to questions regarding sensitivity as described in the white paper, Dr. Parker explained that sensitivity was defined as a significant biological response. Members commented that response variables differ across strains and that the animal model should be chosen based on the most appropriate data to reflect the effects on humans or other species of concern. Dr. Parker noted that multiple strains showed sensitivity at some dose and that dose is important to the screens. She added that there is just variability across the board, which makes it difficult to select a single strain. Another member suggested that sensitivity be further delineated than sensitive versus less sensitive to show when some response occurs.

A member requested further information on why Dr. Spearow does not recommend the use of Sprague-Dawley (SD) rats. Dr. Spearow explained that the narrow genetic base of the population and selection for large litter size lead to correlated responses and lessen sensitivity in SD rats, which is exacerbated by a non-reproducible genotype. Dr. Spearow acknowledged that the white paper does not distinguish between different supplies of rat strains from multiple suppliers.

Another member inquired whether different strains should be considered for different purposes (e.g., general, screening, tier 2). Dr. Spearow responded that tier 2 results are more important and thus should involve a sensitive strain. A member asked whether it was also important to have the most sensitive species in tier 1 screening. Dr. Spearow noted that fertility and gestational response differs across strains. A member asked Dr. Spearow whether he had seen any examples where two species were dosed up to the maximum tolerated dose and one had absolutely no impact at all and one had a dramatic or even a statistically significant impact. Dr. Spearow replied that there are studies showing such differences at specific doses, but he could not say whether they went all the way to the maximum tolerated dose.

A member suggested that EPA conduct comparisons across strain and species for the endpoints of interest. Comparisons should also look for which strain is more appropriate to identify effects on humans according to characteristics such as kinetics, metabolism, physiology, and anatomy. A member recommended adding another source on uterotrophic strain differences (Christian, 1998) that compares SD and certain Wistar strains of rats. A member commented that EPA is looking for the most "appropriate" strain/species, not necessarily the most sensitive.
The members discussed the questions provided by EPA on strain/species.

1) *Are there any mammalian assays being considered for the EDSP in which a specific strain should be used or avoided?*

Several members indicated that they could not answer this question at this time because of a lack of information. A member commented that if EPA selects one strain for validation, it should not be SD CD because the strain is less sensitive for reproductive endpoints. Another member responded that breeders have taken care of concerns with litter size in SD rats and that breeders select all strain/species for large litter size.

Additional comments included the following:

- Using inbred strains could result in less data variability and an overall smaller number of animals.
- Standardization of laboratory techniques can be more important than strain selection.
- The effort it would take to answer this question rigorously would very expensive and very resource intensive. We should have indications that this is truly a major problem before embarking on an effort of this magnitude.
- EDSTAC included in vitro assays specifically to address sensitivity issues, recognizing that in vivo assays may not be the ideal way to get at sensitivity.

2) Should EPA standardize on one strain across all assays?
   a. if different strains are best for different assays
   b. if no strain is preferred for any of the assays

Comments from individual members included the following:

- One strain should not be selected, instead, base selection on whether animals are reasonable, practical, and most appropriate for what would be expected for a specific test chemical.
- Use one standard genetic strain to develop performance standards based on the validation study. EPA could use these standards to set a target for later use of other strains. Standardizing on one strain has advantages of increased reliability, reproducibility, and accuracy.
- If labs want to use a different stock/strain, they must demonstrate good or better results than in the prevalidation study.
- Use the same strain/species in tier 1 and tier 2.
- Initiate pilot studies with highly qualified labs on inbred and outbred strains that involve weak-acting compounds to determine the sensitivity of the strains.
- Once performance standards are established, EPA should allow the lab to choose strain/species. (Performance standards, which would not be completed until after the validation study, would cover a range of responses, be reproducible in different chemical classes, and identify minimum procedural standards in a protocol.)

3) Should EPA require the use of multiple inbred strains? If so,
   a. for which assays?
   b. how many strains?
   c. which strains?
   d. should the total number of animals used in the assay be kept the same as if only one strain were used, or should each strain be tested with the full complement of animals?

Several members commented that this question is difficult to answer, particularly given multiple endpoints, and that the EDMVS should review this question when more is known about the responses of different strains.

Additional comments included the following:

- The use of multiple inbred strains is a recognized practice of reducing overall numbers of animals used in testing, because the use of an inbred strain will have, or should have, less variability in the data.
- Multiple strains increase the size of the assay.

- More powerful results can be obtained if EPA increases the chance of sensitivity through multiple strains.
- Use standard feed across strains to decrease confounding factors.
- There is no apparent justification for using multiple strains.
- Conduct a pair-analysis to determine group size.

4) Is there sufficient reason to switch from rats to a different test species? If so, which species?

A member noted that the only other practical species is mouse, but no persuasive reason has been given to switch. Mice have more problems with reproductive performance. Another member commented that the data on which to base this decision are not available. Another observed that a larger volume of pharmacokinetic and toxicity data are available for rats, which is a compelling reason to continue using rats.

X. Update on Chemicals Used for EDSP's Prevalidation and Validation

Gary Timm, EPA, presented a draft list of reference chemicals to allow comparisons among assays for EDSP tier 1 prevalidation, arranged in tables by mode of action. (As indicated above, copies of the reference chemical tables may be obtained from the docket or EPA website.) He noted that the tables are not intended to be a list of endocrine disruptors. The compounds on the list were chosen because they have produced a well-documented positive response in one or more tier 1 assays by an identified mode of action. They may or may not have been studied in tier 2 assays. Mr. Timm also noted that the tables do not include any negative chemicals. He explained that EDSP staff recognize the importance of including confounders in the reference chemicals but have not yet determined what types of confounders and which negative chemicals to include. He said that in making the selection, EPA will consider the suggestions of negative chemicals previously submitted by members.

As Mr. Timm presented the reference chemical draft tables by modality, members responded to three questions as summarized below. Members also noted some omissions in the tables and offered to provide Mr. Timm the relevant information to revise the tables.

1) Does the EDMVS agree with EPA's decision to limit consideration of data to the studies conducted in the EDSP validation program, EPA Office of Research and Development laboratories, the OECD assay validation program, and for the adult male, the U.S. chemical industry laboratories?

Mr. Timm clarified that one reason for this limitation is to ensure that the data being compared are generated from the same protocol.

A member commented that there may be published industry studies for haldol and reserpine that would provide additional data for the female pubertal assay. She explained that the studies used the same protocol as each other, and the protocol is similar to the one currently being considered by EDSP.

2) *To ensure that the assays selected to comprise the tier 1 battery cover the known modes of action in a comprehensive, complementary, and efficient fashion, EPA has grouped the chemicals used in prevalidation into five modes of action. How many chemicals should be selected to compare assays across modalities?*

A member acknowledged that it would be costly and time consuming to test all of the compounds in the in vivo assays, but suggested that a more complete comparison could be done with the in vitro assays. Another member agreed, commenting that it might be useful to generate data for these in vitro tests on most or all of the chemicals, regardless of their modality.

A member suggested that initially, the chemicals should be selected based on first principles to determine whether the assay detects chemicals that act by the specified mode of action, then whether it works across the intended classes of chemicals, then whether it is sensitive, and then whether it is specific. Mr. Timm agreed with the general approach, but noted that it can be difficult to be sure that a given chemical has a pure mode of action and is not multi-modal. He also noted that in addition to validating individual assays, the EDSP must compare among assays to select the battery. The challenge is in choosing a sufficient number of chemicals to provide the data to select among the assays without requiring so many chemicals as to be cost prohibitive.

A member suggested focusing on the critical comparisons among the assays and making sure that the overlap of chemicals between those pairs or sets of assays is sufficient. Another member added that it would be useful to frame the questions that will have to be answered in the end in order to select the battery.

The EDMVS agreed that a small group of members and other experts would work with EDSP staff to develop a prioritized selection of reference chemicals, focused on critical comparisons among the assays. The results of the small group's efforts will be shared with the full EDMVS for comment.

3) *What additional chemicals should be chosen for specific assays during validation to facilitate comparisons?*

Comments from individual members included the following:

- A compound such as estradiol should be included in the steroidogenesis assay given that it has a known feedback modality.
- EPA should refer to the ICCVAM report "ICCVAM evaluation of *in vitro* test methods for detecting potential endocrine disruptors: estrogen receptor and androgen receptor binding and transcriptional activation assays" (NIH Publication No. 03-4503, May 2003), published in June for the recommended minimum sets of chemicals for AR and ER binding assays.
- In the ICCVAM report, most of the negative chemicals are drawn from the other modality (estrogen vs. androgen receptor binding), so that there are at least 25 percent negatives for each of the in vitro assays.
- For thyroid modality, EPA should consider examining other modes of action rather than just including an inducer.

- For neuroendocrine, only atrazine is being compared across multiple assays. EPA should consider including additional cross-assay chemicals.
- For neuroendocrine, transmitter agonists, such as a serotonin analog, a dopamine analog, or norepinephrine, should be considered.
- The negative chemicals selected should include nonspecific toxins.

XI. **Avian Detailed Review Paper**

Dr. Touart presented a summary of the *Revised Draft Detailed Review Paper for Avian Two-Generation Toxicity Test*. (As indicated above, copies of slides from Dr. Touart's presentation may be obtained from the docket or EPA website.) He described the two test species – Japanese quail and bobwhite – and listed the advantages and disadvantages of each. He then reviewed the exposure considerations, routes of administration, dosing options, statistical approaches, fitness endpoints, physiological endpoints, and biochemical measures examined in the paper. The candidate protocols reviewed in the paper are the short-term life cycle test (proven breeders), life cycle tests, and the two-generation test. The recommended protocol uses the Japanese quail as a test species and includes eight fitness endpoints as well as endocrine endpoints for gross morphology and histopathology, developmental landmarks, and plasma and fecal/urate hormones. The exposure protocol will be decided upon based on the results of the avian dosing study.

Dr. Touart also outlined the data gaps, implementation considerations, and areas for future research identified in the paper. He listed the issues and concerns that remain for developing an optimal protocol:

- Animal usage.
- Value added of two-generation versus one-generation assay.
- Japanese quail sensitivity.
  - Lack of avian assay in tier 1.
- Time delay in validation (and costs).
- Linkage with existing avian testing framework.

Discussion

The members discussed the 3 questions provided regarding the detailed review paper.

1) *Does the EDMVS agree that a two-generation method recommended with Japanese quail is appropriate?*

Comments made by members in agreement included the following:

- A two-generation method is clearly needed in the study.
- A two-generation method will lead to time and cost savings.
- A two-generation method is more practical.
- Bobwhite quail are more excitable than Japanese quail.

Some members suggested improvements and considerations to address before moving forward:

- For thyroid studies, look at T4 in the gland as opposed to circulating thyroid; T4 in the gland may be a true biomarker of exposure.
- Japanese quail is a fine choice, but EPA needs to review data on piscivorous species.
- EPA should prioritize endpoints because labs cannot handle the entire large set.
- Diet should be assayed for components of grain and chemical contaminants (e.g., pytoestrogen) and limits established for contaminants in feed.
- Consider possible differences in strains of Japanese quail.

In response to a question, Dr. Touart explained that EPA is considering including thyroid and gonad histology as potential endpoints.

2) *Does the EDMVS agree that for purposes of evaluating potential adverse consequences of putative endocrine disrupting chemicals in Tier 2, fitness endpoints should be emphasized more than mechanistic endpoints? Does the EDMVS have recommendations for balancing the inclusion of mechanistic endpoints?*

Comments in favor of emphasizing fitness endpoints included the following:

- Mechanistic endpoints are not required.
- The relationship between fitness and endocrine modes of action are more apparent.
- EPA should use a broad biological interpretation of fitness rather that a narrow definition around risk assessment, which focuses on the population to the exclusion of individual animals.

Comments supporting the use of mechanistic endpoints included the following:

- A sufficient number of mechanistic endpoints are necessary to establish relevance.

Members shared additional comments on endpoint selection:

- EPA should consider endpoints based on reasonability and practicality.
- Factors to consider include balancing relevance to risk assessment, technical difficulty, and ability to validate.
- Fitness and mechanistic endpoints cannot be delineated sharply.
- Collect a broad range of fitness and mechanistic endpoints, then select the ones that are most useful and reproducible.
- Characterize the egg composition in the chosen quail strain(s), particularly for fatty acid profiles and fat soluble vitamins, which can vary markedly based on diet. Maintaining consistency between the F1 and F2 generations will help to determine whether results are due to the test chemical or due to an alteration in the quality of the egg because of some other reason.
- Consider effects of group versus individual housing that can impact maturation of the animal.
- Literature exists on sensitivity to testosterone on the vocalization systems.
- Add an endpoint for comb size that is measured at the end of the assay.

3) *Does the EDMVS have suggestions to improve the DRP?*

Suggestions from members included the following:

- Conduct one more round of literature review to catch items missed in earlier reviews, such as test protocols and endpoints.
- Make sure that statistical power in the endpoints demonstrates true variability and statistical significance.
- Bring in experts from outside the risk assessment and toxicology field (e.g., wildlife biologists).
- Include dialogue about dose selection and measures of overt toxicity in the assay.
- Include description of usefulness of birds for risk assessment.

In response to questions from members, Dr. Touart clarified that the second generation begins six weeks after egg laying to ensure a full egg cycle. A member inquired as to whether the chemical selection for validation studies has been finalized. Dr. Touart responded that the selection is not finalized. Dr. Touart stated that EPA will have more information for the EDMVS by next spring after a side-by -side quail study on toxicity testing and the dosing study are completed and available. He explained that EPA will revise the draft DRP based on comments, forward to OECD by next spring, and revise again based on additional comments.

XII. **Issues Related to the Pubertal Assay**

Mr. Kariya explained that EDSP staff had hoped to provide the EDMVS the data from the two pubertal studies for discussion at this meeting. However, despite a great effort, the staff and contractors were not able to complete all of the analysis given the large amount of data. EPA plans to provide the data for the December meeting, but the staff wanted to introduce some of the issues at this meeting to help prepare EDMVS members for their review and discussion of the studies.

L. Earl Gray, EPA, shared a presentation summarizing these issues. (As indicated above, copies of slides from Dr. Gray's presentation may be obtained from the docket or EPA website.) He reminded meeting participants of the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) recommendations regarding the tier 1 screening battery, noting that the committee recommended that the male pubertal assay be developed and evaluated for screening. He reviewed the history of the current protocols and suggested some questions that EDSP may want to pose to the EDMVS for future discussion:

- Given the number of chemicals run in more than one lab, is a formal interlaboratory study still needed?
- Is the pubertal male assay sensitive enough to replace the Hershberger assay in tier 1?
- Should we evaluate the adrenal or other endpoints in more detail?
- What mechanisms of action need further evaluation to establish the sensitivity of the assay?
- Should we be running concurrent dose-range finding studies?
- Can we reduce the sample size in these assays?

He noted that the data to be presented at the December meeting will help in answering several of these questions. He then outlined the chemicals investigated by the EPA contractors and those in published studies using the pubertal female assay.

In regard to the effect of different rat strains on assay results, Dr. Gray noted that the results of one study completed by TherImmune indicated a difference in response between two strains with one chemical in the males, and the results of the OECD validation of the uterotrophic assay indicated no differences among strains. The OECD work also found that the studies were robust across variations in diet. Dr. Gray commented that the importance of these factors remains to be determined.

Dr. Gray presented some of the data from the TherImmune study on the pubertal female assay in 2000, noting that a significant response was detected with many of the chemicals for the various endpoints. He commented that changes in hormone levels and organ weights are observed in the males as well. He then listed the chemicals investigated by the EPA contractors and those in published studies using the pubertal male assay.

Dr. Gray shared data from various studies to illustrate the following:

- The ability of the pubertal assay to detect effects on the age at preputial separation with less potent chemicals.
- The better diagnostic ability of the female assay than the male assay for xenoestrogens.
- How different endpoints correlate with body weight.
- The correlation of dose response curves despite laboratory and strain differences.
- The significant results obtained in the TherImmune 2000 study for various endpoints using seven different chemicals.

Observing that the ultimate question will be which assays to include in the battery, Dr. Gray presented some data comparing the Hershberger assay, the intact male assay, and the male pubertal assay. He commented that that the assays under consideration should be evaluated using weak antiandrogens.

In regard to the question of whether EPA should run dose-range-finding studies as part of the validation for the pubertal assays, Dr. Grey proposed following the model of the dose-range-finding study conducted for the in utero study and presented to the EDMVS in 2002.

In closing, Dr. Gray suggested some additional endpoints for possible inclusion during assay validation or follow-up tier 1.5 studies.

Discussion

Several members inquired about dose selection, including how the researchers set dose levels and conducted dose level studies. Dr. Gray responded that EPA sets dosing levels comparable to those in the literature and that some dosing is blinded for dose and for chemicals. Some members encouraged EPA to conduct a dose setting study and incorporate dose range findings in the protocol. Some commented that if EPA does not conduct this study, the agency should choose chemicals where doses are already known or conduct dose range finding studies in multiple labs. Another member added that dose selection is critical and should not affect endpoints.

A number of members also commented on issues related to the diet of the animals in the assay. Members recommended that EPA conduct a critical review on the possible confounding effect of phytoestrogen in diets on the results of the studies. A member emphasized that it is important to

know diet phytoestrogen levels before formal validation studies. Dr. Gray noted that this could be a white paper or a study. Noting the time and cost issues of conducting a diet study, a member suggested that EPA should incorporate diet in the study design rather than analyze after the study.

Members suggested several additional endpoints, such as testes weight, adrenal function, and pancreas study (for obesity-related issues). Dr. Gray explained that reproductive organ weight is not associated with the general growth of the animal and that although data on the pancreas were not collected, if EPA decided it was useful, the agency would learn more and solicit experts in that area.

Members offered the following additional comments and suggestions:

- EPA needs to address criticisms of study design in development of a standard protocol.
- EPA needs to formally document intralaboratory studies.
- Do not need to use numerous different chemicals in the assay.
- Explore other pathways such as dermal absorption through the eye and inhalation, and consider the constant exposure to humans found in homes.

For the December EDMVS meeting, members requested a presentation on the intact adult male assay in order to compare alternatives, including the pubertal male and Hershberger assays.

XIII. **Steroidogenesis Assay Optimized Protocol**

Carol Sloan, RTI International, presented a summary of the optimization of the sliced testis steroidogenesis assay. (As indicated above, copies of slides from Ms. Sloan's presentation may be obtained from the docket or EPA website.) The optimization was conducted in two phases. Ms. Sloan briefly summarized the design and results of the first phase, noting that they were presented more fully at the June EDMVS meeting. She explained that the second phase was the primary experimental phase and outlined the study design. The factors to be tested were divided into four groups: incubation conditions, testis preparation, sampling time, and characterization of variability. She presented the results of the experiments on the groups of factors and then summarized the optimal factors as determined through the study:

- 11-15 week old rats should be used
- Medium199 without phenol red is the preferred culture medium
- Gaseous atmosphere of 5% CO2 / 95% O2
- Optimal temperature for incubation was approximately 36ºC
- Optimal vessel for incubation was the scintillation vial
- Optimal shaker speed was approximately 175 rpm
- Optimal media volume was approximately 4-5 mL
- Optimal hCG concentration for stimulation was 0.08 to 0.1 IU/mL
- Optimal fragment size varied from 100-200 mg
- Time delay before the start of the incubation should be no more than 1 hour from
- the time of testicular tissue removal
- The solution that the testes are collected in may be either cold DPBS or cold M-

- 199
- The sample aliquot size removed for testing should be around 0.5 mL

In closing, Ms. Sloan shared several "points to ponder:"

- All of these assays were performed on control rat testes.
- No assays included any test chemicals except for possible vehicles that may be used in the prevalidation and validation assays.
- It may be necessary to measure specific viability (Lactic dehydrogenase (LDH) is not cell specific).

Discussion

In response to questions from members, Ms. Sloan clarified that testes were kept in separate vials to compare animal to animal. She also explained the purpose of the LDH assay. Cytotoxic chemicals will cause cell death and a reduction or cessation in testosterone production, which could be interpreted as a positive response. LDH is released when cells die, thus elevated LDH levels are an indicator of cytotoxicity. When testosterone production decreases in the presence of increased LDH, the decrease in testosterone will be attributed to cell death rather than inhibition of steroidogenesis. Some members commented that LDH was an inappropriate measure of Leydig cell viability because it is indicative of general cell toxicity and is not specific to Leydig cells. In response to this concern, Ms. Sloan discussed a cell staining technique to detect 3-beta-HSD as an alternative to the LDH assay for cell death. However, comments were made that this was an even more problematic approach because the procedure measures the presence of the enzyme 3-beta-HSD which remains active after cell death. Thus, this is not an indicator of cell viability. EPA staff believe there will be few toxicants that kill Leydig cells that do not also injure other cell types.

Ms. Sloan noted that the major change in this study from the published literature involved two major variations: the finding of an optimal temperature of 36° instead of 34° used in the literature; and an optimal air composition of 95% oxygen and 5% carbon dioxide. She added that this study, conducted with a combination of literature values, will aid EPA and RTI decision making. A member inquired about the reproducibility of the assay. Dr. Sloan replied that the reproducibility is "decent."

Member reservations about the assay included the value of the assay as compared to others given the incremental gains and that the modest response in the signal/noise ratio versus the baseline could limit the ability to detect change. One member suggested that rather than using a full factorial design, the study could use incomplete data sets to identify the major factors and then focus on them. He noted that this sort of "leap frog" approach is used in other fields to move more quickly to a decision.

XIV. **Presentation and Discussion on Steroidogenesis Prevalidation Study Plan**

Mr. Timm presented the prevalidation study plan for the sliced testes assay. (As indicated above, copies of slides from Mr. Timm's presentation may be obtained from the docket or EPA

website.) He summarized the concerns and advice expressed by EDMVS members at the June meeting. He commented that EPA considered these concerns and advice in redesigning the prevalidation program. Mr. Timm then outlined the redesigned program. Key points of the program include the following:

- The program will focus only on prevalidation; validation will be a separate work assignment.
- The program will involve a lead laboratory and three participating laboratories.
- Responsibilities of the lead laboratory will include:
    - baseline and testes variability study,
    - test of positive control,
    - cytotoxicity studies,
    - multichemical studies, and
    - training of personnel at participating laboratories.
- The participating laboratories and lead laboratory will conduct the baseline studies and positive control studies in triplicate.

Mr. Timm reported that the estimated completion date for the prevalidation is April 30, 2004.

Discussion

Members' key comments on the steroidogenesis prevalidation study plan related to issues of interlaboratory variability, training, and cytotoxicity. Regarding variability issues, members encouraged EPA to be aware of variability among testes, conduct multichemical studies in more than one laboratory to prevent bias, conduct tests between laboratories as part of prevalidation, and employ a rigorous procedure for determining materials. One member commended EPA for including chemical characterization. Members also advised that EPA should train laboratory supervisors as well as the next lower level of staff at lead laboratories and other laboratories.

While some members believed that cytotoxicity is not a significant issue, others noted that cytotoxicity issues need to be worked out or every chemical will decrease testosterone as Leydig cells are killed. A member added that if LDH results are highly variable, interpretation of other results becomes more difficult. Members suggested that EPA can address this issue with stains or other methods.

XV. **Public Comment**

At the conclusion of the deliberations on the first and third day of the meeting, members of the public were given the opportunity to provide comments. Mr. De Morgan indicated that each person's comments would not be captured verbatim in the meeting summary, but rather just briefly summarized. He encouraged all to submit their comments in writing to Ms. Smith for inclusion in the EPA docket and posting on the website. Slides of some of the individuals' comments may be obtained from the docket or EPA website.

Monday, August 18, 2003

Richard Becker, American Chemistry Council (ACC)

Dr. Becker presented comments from the ACC on the Steroidogenesis Sliced Testes Assay. The ACC believes that underlying problems with the cell preparation in the assay exist and has strong concerns with the ability of the assay to detect cytotoxicity, including that the use of the 3*beta*-HSD histochemistry is not appropriate for use as a Leydig cytoxicity assay. The ACC questions whether the assay should proceed given the inherent limitations and recommends that the EPA consider devoting additional resources to the 15-day intact male assay, which the organization considers to be a sensitive, specific, and mechanistic-based screen.

Tilghman Hall, Bayer CropScience for Crop Life America
Dr. Hall outlined several weaknesses in the analysis, results, and methods of the VTG methods comparison. Dr. Hall offered two alternative VTG methods that may prove easier to validate, such as gel electrophoresis with Western blot and plasma alkaline-labile phosphate. She also presented several questions to consider in evaluating the VTG endpoints, including purpose, relevance, and level of effort, as well as development considerations if the endpoint is deemed essential.

Katie Holmes, BASF Agro Research for Crop Life America
Ms. Holmes discussed the fish reproduction comparative protocols. She presented several general comments on the use of an apical assay to determine mechanism of action, selection of endpoints such as egg production, and support for a 14-day spawning protocol. In responding to questions put before EDMVS, she noted that the suitability of the assay as a tier 1 screen depends on the purpose of the assay and that the 14-day egg production assay is sufficient for the detection of effects.

Reinhard Fischer, Bayer CropScience for Crop Life America
Mr. Fischer provided comments on the Avian Two Generation Toxicity Test Detailed Review Paper. He remarked generally on the thoroughness of the paper and commented that more current data could be identified and that resources needed to complete the study should be included. He noted that the study objectives should be clarified, the most important of which should be identification of a safe dose level. Regarding test species and exposure, he recommended the use of Japanese quail with continuous dietary exposure. He also commented that the NEOC approach for the study design is preferred and that the assays should examine only a selection of the most relevant, robust, and sensitive endpoints.

Wednesday, August 20, 2003

Richard Becker, American Chemistry Council (ACC)
Regarding the sliced tested assay, Dr. Becker encouraged EPA to develop a predictive model to optimize the system and to use a mechanism-specific assay. He noted a correction to the chemicals table to show that alkylating agents are not specific Leydig cell toxicants. Dr. Becker also offered comments on the EDMVS process, suggesting that EPA reconsider the structure of the committee to add working groups of members and outside experts to address specific assays. He also complimented EPA's hard work and handling of the iterative nature of the process.

David Paulsen, Clark County Water Reclamation District, Las Vegas, NV

Mr. Paulsen expressed concern with the body of literature and statistical treatment for VTG studies, which impacts industry because they are required to report all data from all tests. He recommended increased efforts on statistical analysis, including adding a statistician to the EDMVS.

Jim Pletel, Hampton Road Sanitation District
Mr. Pletel provided comments in five areas:

- EPA should allow wider participation in conference calls and hold meetings in the Washington, D.C. area near the headquarters of interest groups.
- EDMVS members are not clear on how data are going to be used to make decisions, which influences their input.
- Uses of data include regulating chemicals before they reach the environment, regulating broad use of chemicals, regulating wastewater effluent, and assessing compliance. EPA should consider the boundaries of the use of endocrine disruptors to prevent inappropriate use.
- Refine protocols to increase ability to look at biological significance.
- Follow EPA guidance on data quality and incorporate into the protocols.

XVI. **Agenda for Next Meetings**

Future Meeting/Teleconference Topics

Ms. Smith listed the meeting topics planned for the December and spring EDMVS meetings.

December 10-12, 2003

- Pubertals male and female
- In Utero Through Lactation
- Update on Androgen Receptor binding
- Aromatase
- Update from OECD Fish Drafting Group
- Update on activities regarding *in vitro* fish assays
- Update on reference chemicals
- 14-Day Adult Intact Male
    - Presentation of EPA data (tentative)
    - Suggested: 14-Day Adult Intact Male presented by industry

Spring 2004 (Dates to be determined)

- Amphibian DRP
- Amphibian Screen transferable protocol
- Fish Screen multi-chemical results
- Fish Screen Phase 1B (OECD)
- Mysid Transferable Protocol

Miscellaneous

The group spent a few minutes at the end of the meeting suggesting approaches that could assist in management of the documents as well as making more efficient use of their time. In particular, the group suggested the following:

- Take 'Draft' watermark off documents (put as header) as it makes it hard to print.
- Use more teleconferences.
- Include executive summaries for reports, DRPs, and other documents.
- Present/develop more assay comparisons.

Members were thanked for their time and the meeting adjourned at 2:45 p.m.

Attachments: A. EPA Reflections
B. Supporting Materials for the EDMVS

Attachment A

EPA Reflections

Reflections, Next Steps

Before the meeting adjourned on August 20, EPA staff presented the following lists summarizing the key points and potential action items they had drawn from the subcommittee's discussions on fish assays, reference chemicals, and strain/species.

Fish Assays

- Should EPA move forward with assay?
  - Some voices yes, others require more convincing.
- Both VTG method survey and fathead minnow assay comparison need revision.
  - Statistical analysis and data presentation.
- VTG is an important endpoint for the screen but requires major improvements.
- Some support for fecundity as an endpoint, but recognize as apical not mechanistic.
  - Requires more convincing justification.
- Mixed comments about inclusion/exclusion of other endpoints.
  - Thyroid relevant endpoints suggested to be added.
  - Histopathology needs more information.
- Power analysis for various endpoints should be presented.
- Need to include non-specific toxicants and establish criteria for concentration setting (e.g., MTD).
- Some support for narrowing field.
  - Fathead minnow recommended.
  - More convincing rationale needed for assay choice.
- Explore whether more effort should be given to in vitro fish assays.

Following the presentation of these reflections, a member suggested some specific next steps in regard to deciding whether more effort should be given to in vitro assays:

- Bob Combes will provide EPA a summary of activities regarding in vitro assays in Europe.
- EPA will provide additional information to EDMVS members on current work by various entities on in vitro assays.
- EPA will decide whether more effort should be given to in vitro assays and report the decision to EDMVS.

Dr. Touart noted that the EDMVS did not thoroughly discuss the original questions posed by EPA in regard to the fish assays. He asked members to submit written comments to Ms. Smith.

Reference Chemicals

- Message Heard:
  - Identify main comparisons to be made (e.g., intact male, pubertal male).
  - 5-8 positives per mode of action should be adequate.

- Fill out relevant mode of action completely for in vitro assays. Consult ICCVAM report.
- Give attention to selecting negatives for each assay.

Strain/Species

- Should certain strains be preferred or avoided for individual assays?
  - Unanswerable right now.
  - Would take an enormous effort to determine how important strain is relative to other factors.
  - No "flashing red lights" indicating there is a problem with EDSP assays, but….
  - Possibly avoid Sprague-Dawley (CD)? Take up this question again after examining the incoming pubertal data (Dec 2003 meeting).
  - In vitro assays will help address concerns about sensitivity of the Tier 1 battery so it is not absolutely critical to find the most sensitive strain in Tier 1 assays.
- Should EPA strive for uniformity in strain across all assays?
  - In prevalidation and validation, yes.
  - In guidelines, not sure.
  - In prevalidation/validation, use the same strain in Tier 1 and Tier 2 so that correlations between tiers are more easily interpretable.
  - No advice on which strain to choose.
- Should we use multiple (inbred) strains in (some/all) assays?
  - This is a good goal to work toward because it has the potential to reduce animal use.
  - It is not an unusual practice when only one endpoint is involved, but it is currently too difficult to implement for assays with multiple endpoints.
  - Development of this approach should not hold up implementation of EDSP.
- Is there reason to switch from rats?
  - A data-based answer specific to the EDSP assays is not possible: no data were presented.
  - In general, mice have more reproductive problems than rats and this would make endocrine disruptor testing more difficult. However, if someone develops data showing equivalence to rats in a specific assay (e.g., uterotrophic), there is no reason to reject mouse data out of hand.
  - In general, the greater availability of detailed ADME data and other relevant information for rats is a compelling reason to stay with rats.

In discussion following the presentation of these reflections, a member suggested there were some "red lights" in regard to the EDSP assays, such as logistical problems and difficulties of analysis. Another member commented that the wording regarding possibly avoiding Sprague-Dawley rats was stronger than he would support. He said he had seen no data to support avoiding SD rats for tier 1. A third member commented that the group had in fact seen data showing that SD CD rats are stable. She noted that they are a widely used strain, are kept standardized for litter size, and may be the only group that does not have genetic drift. She requested that data regarding these points be added to the white paper to supplement the analysis.

Attachment B

Background Materials for the EDMVS
August 18 - 20, 2003 Meeting
Docket – OPPT-2003-0027
Website: http://www.epa.gov/scipoly/oscpendo/

1. General Procedural

- Proposed Agenda
- June 5 – 6, 2003 EDMVS Final Meeting Summary
- EDMVS Work Plan, Revised

2. Fish Screening Assays

- Report on Survey of Vitellogenin Methods in Fathead Minnow
- Report on Survey of Vitellogenin Methods in Zebrafish and Medaka
- Report on Comparative Evaluation of the Fathead Minnow
- OECD Phase 1A
- Questions for the EDMVS regarding the fish screening assays

3. Strain and Species White Paper

- "White Paper on Species/Strain/Stock in Endocrine Disruptor Assays"
- Questions for the EDMVS regarding strain/species

4. Chemicals Used for EDSP's Prevalidation and Validation

- Draft Spreadsheet of Chemicals Used for EDSP's Prevalidation and Validation

5. The Avian Assay (Tier II)

- The Avian Detailed Review Paper
- Questions for the EDMVS regarding Avian Two-Generation Toxicity

6. Pubertal Male and Female Assays – Issues Related to the Pubertal Assay (References Only)

- "Endocrine-Disrupting Chemicals: Prepubertal Exposures and Effects on Sexual Maturation and Thyroid Function in the Male Rat. A Focus on the EDSTAC Recommendations"
- "Endocrine-Disrupting Chemicals: Prepubertal Exposures and Effects on Sexual Maturation and Thyroid Activity in the Female Rat. A Focus on the EDSTAC Recommendations"

7. Steroidogenesis Assay

- Report on Steroidogenesis Assay Optimized Protocol
- Steroidogenesis PreValidation Study Plan